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13. ABSTRACT *(Maximum 200 words)*
Using MCF7 cells (a human breast carcinoma cell line which is estrogen receptor positive), our lab built a system in which transfection of either FGF4 or FGF1 into this cell line allows cells to form progressively growing tumors in athymic nude mice without estrogen supplementation. The overexpression of FGFs also forms micrometastases in different organs. The hypothesis to be tested is that FGFs are also capable of increasing the malignant potential of breast epithelial cells that are not as far along on a progression pathway as MCF7 cancer cells. We chose MCF10A cells, which is a spontaneously immortalized mammary epithelial cell line to study this hypothesis. The background screening of this cell line, at mRNA level, indicates that this cell line expresses high level of FGF2 (+++), also expresses FGFR1 (+), barely detectable FGFR2 (+\-), and does not express FGF1, FGF4, and FGFR3. At the protein level, we demonstrated that a high amount of FGF-2 protein exists in MCF10A cell lysate, but barely detectable levels are present in the conditioned media. We transfected MCF-10A cells with FGF1 and FGF4. Using RNAse protection assay, we obtained transfected clones that express FGF4. Using Southern blot assay, the data shows the FGF4 and FGF1 genes are integrated into the MCF-10A genomic DNA.

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FOREWORD

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INTRODUCTION

The members of the fibroblast growth factor family of ligands and receptors have been implicated by a number of studies to be involved in the processes underlying the development of breast hyperplasia, neoplasia, and metastasis. We have observed expression of FGF receptors and FGF-1 and FGF-2 in a majority of tumors and overexpression of FGF receptors in a number of breast cancer cell lines. Thus, the possibility exists for a dynamic interaction between tumor and stromal elements involving autocrine and/or paracrine effects due to the production of these proteins. The interruption of this dynamism may lead to reduced tumor growth or spread by interfering with angiogenesis or tumor cell division.

We have taken the approach of using cDNA expression vectors to overexpress growth factors or growth factor receptors in MCF-7 cells to assess the effects of this constitutive overexpression on estrogen-dependent growth *in vivo* or *in vitro*. In this way, if a progression is observed, it can be assigned to a particular gene or combination of genes. Such a finding would therefore provide a focal point around which one can search for the existence of similar or identical growth factors or receptors in subtypes of human breast tumors. The success of the approach would also provide an experimental model system for the testing of more specific therapies aimed ultimately at interrupting the autocrine or paracrine effects of the identified signal transduction pathway in that subtype or subtypes. This approach also provides cell lines that are likely to be one step further along a progression pathway. These cells can then be used as a new starting point to identify what additional changes might result in a further progression that might not be observed if these manipulations were performed on a less progressed cell line. This was just the case when MCF-7 cells were transfected with expression vectors for either FGF-4 or FGF-1. The results of these studies indicate that overexpression of either gene alone would confer an estrogen-independent phenotype *in vivo* that included resistance to the antiestrogen tamoxifen. Both types of transfectants also showed a dramatically increased ability to form micrometastases.

Given these apparently unique effects of FGF overexpression in this model system, we consider it reasonable to focus on elucidating the mechanisms responsible for its effects and when in a progression pathway it effects are capable of being exerted. MCF-10A cells are a unique system for studying the mechanisms of mammary epithelial cell transfection by cellular proto-oncogenes and growth factor genes. These cells are a near-diploid cell line derived from a population of normal human luminal mammary epithelial cells. They are a spontaneously immortalized cell line. MCF-10A cells have the characteristics of a normal breast epithelium by the following criteria: (a) lack of tumorigenicity in nude mice; (b) three-dimensional growth in collagen; (c) growth in culture that is controlled by growth factors; (d) lack of anchorage-independent growth; and (e) dome formation in confluent cultures. They therefore represent a useful system to test the hypothesis that FGFs are also capable of increasing the malignant potential of breast epithelial cells that are not as far along on a progression pathway as MCF-7 cancer cells.

We propose to transfect the MCF-10A cell line with *lac Z* and FGF-1 or FGF-4 expression vectors to determine if cells at an earlier stage in the breast cancer progression pathway can acquire a

tumorigenic and metastatic phenotype as a result of FGF overexpression. This report covers work done in the first year of the project.

BODY

1.FGF and FGF receptor expression in MCF-10A cells. Using RNAse protection assays, we checked the possibility that MCF-10A cells already express FGF-1 or FGF-4. We found that this cell line does not express detectable levels of any of these RNAs. However, with the RNAse protection assays we did observe low but detectable levels of RNA for FGFR-1 and FGFR-2 (Fig.1). A separate RT-PCR analysis indicated that it is the FGFR-2 exon IIIB isoform that is exclusively expressed.Therefore, they should be capable of responding to FGFs if an appropriate *in vitro* or *in vivo* systems allowing detection of such a response can be established. However, with RNAse protection assays, we did not detect mRNA for either FGFR-3 or FGFR-4 (Fig.2).

FGF-2 is a *Mr* 16,000-18,000 polypeptide mitogen that has been purified from a wide variety of tissues. Although originally reported to stimulate the proliferation of cells only of mesodermal or neuroectodermal origin, FGF-2 has since been shown to be mitogenic for ectodermally derived cells such as human epidermal keratinocytes and melanocytes (1) . FGF-2 does not possess a signal

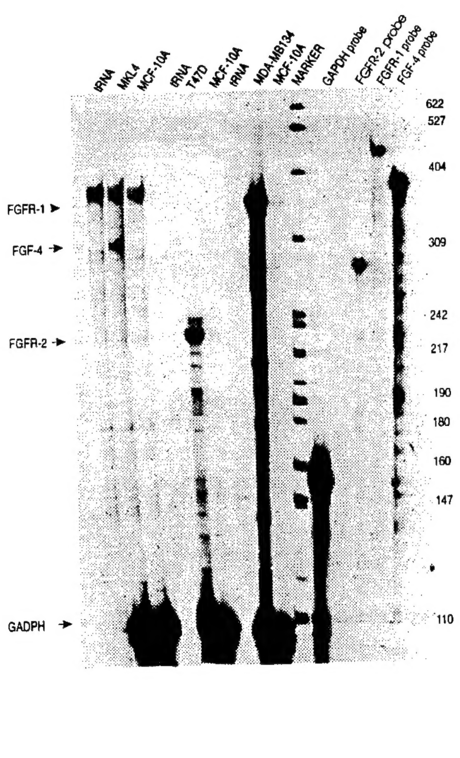


Figure 1. RNAse protection assay demonstrating expression of FGFR-1 in MCF-10A cells

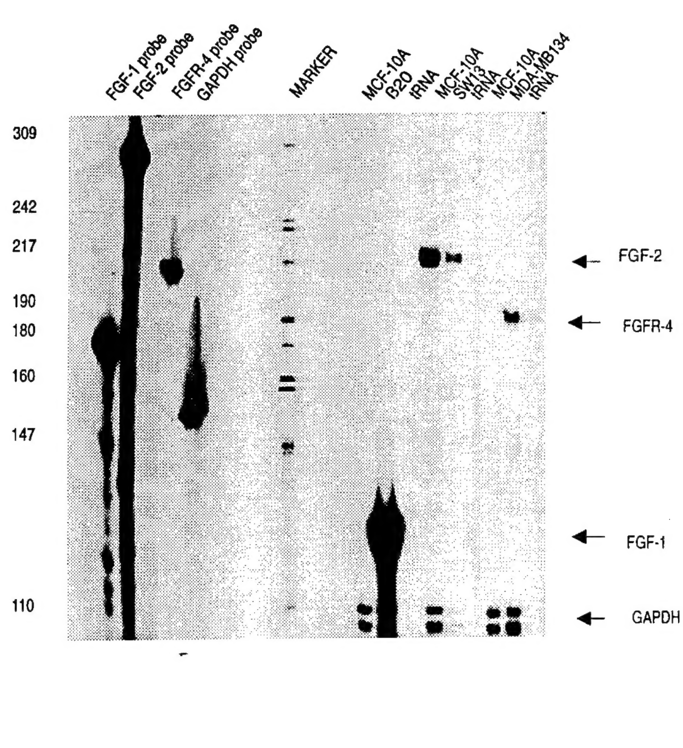


Figure 2. RNAse protection assay demonstrating expression of FGF-2 in MCF-10A cells

peptide and was mainly detected in cell extracts and extracellular matrix preparations (2,8. As part of the survey of FGF expression in MCF-10A cells, we did a RNAse protection assay for FGF-2 in this cell line. We observed high levels of mRNA for FGF-2 in this cell line, comparable to the levels seen in SW13 cells that were used as the positive control in the experiment (Fig2).Using a Western blotting assay, we found that FGF-2 can be detected using as little as 30 ug of MCF-10A cell lysate when a rabbit anti FGF-2 antibody is used (Fig3). Using an ELISA for FGF-2, we found FGF-2 present in the serum-free conditioned medium from MCF-10A cells at the approximate limit of detection for the assay (120 pg/ml/0.73million cells). Since this value falls below the K_d of the high affinity FGF receptors, this amount is less than that needed to observe activation of FGF receptors.

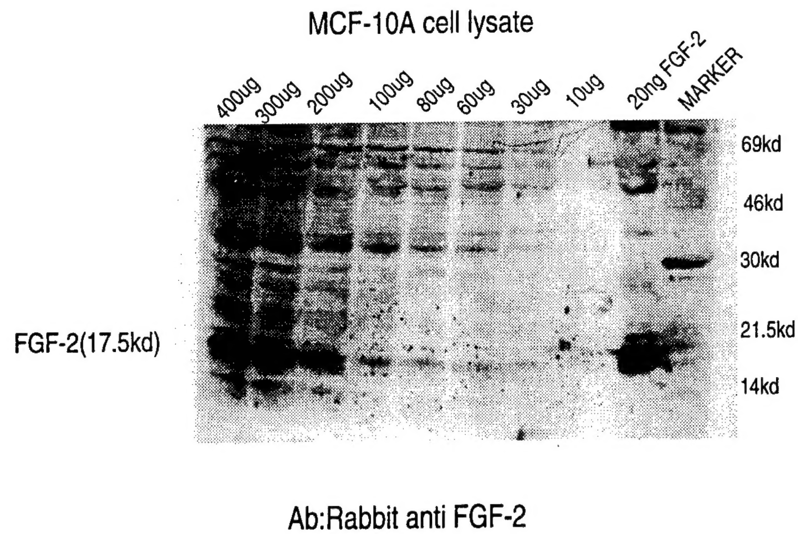


Figure 3. Western blot showing FGF-2 protein in the MCF-10A cell lysate

Although SW13 cells are derived from an adrenal cortical carcinoma, they are like MCF-10A cells in that they do not exhibit many of the characteristics associated with transformed cells. They are nontumorigenic in nude mice and they fail to form colones in soft agar without the addition of exogenous FGFs. However, transfection of SW13 cells with FGF-4 confers these cells with the ability to grow in soft agar and to form tumors in nude mice (3). This result shows the requirement for extracellular localization of the overexpressed FGF for full transforming activity. This result provides an additional rationale to overexpress extracellulaly localized FGF-4 and FGF-1 β (amino acids 1-154 of FGF-1 fused at the amino terminus to the secretory signal sequence of FGF-4) in MCF-10A cells.

*2. **Anchorage dependent growth assay***: The growth of MCF-10A cells is dependent upon the addition of hydrocortisone, insulin, cholera toxin, and EGF to the growth media. Using different treatments, we confirmed that MCF-10A cells depend upon the presence of EGF for *in vitro* growth. In the absence of EGF, the addition of FGF-1, FGF-2, and FGF-4 can increase cell growth but not to the same levels seen with EGF addition. These results suggest that (a) after FGFs are transfected into MCF-10A cells, MCF-10A cells may have reduced EGF dependence and (b) an EGF-free media might be used as selective media for the selection of cells sucessfully transfected with FGF cDNAs.

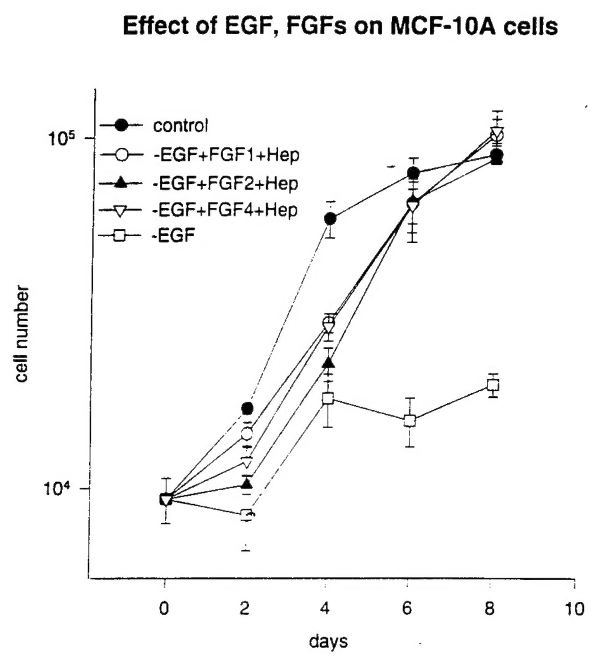


Figure 4. Anchorage-dependent growth assay of MCF-10A cells. Control is the complete media for MCF-10A cells. Either FGF1, FGF2 or FGF4 is 30ng/ml. Heparin in the media is 50ug/ml.

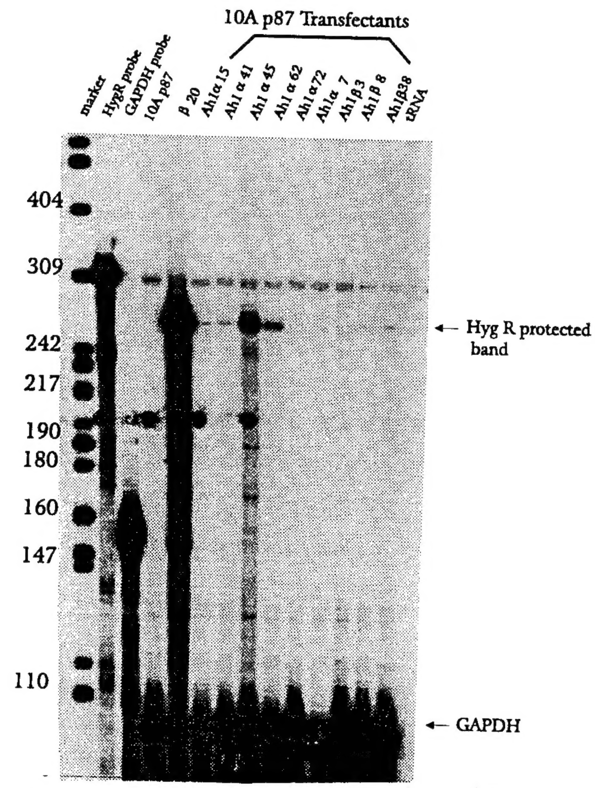
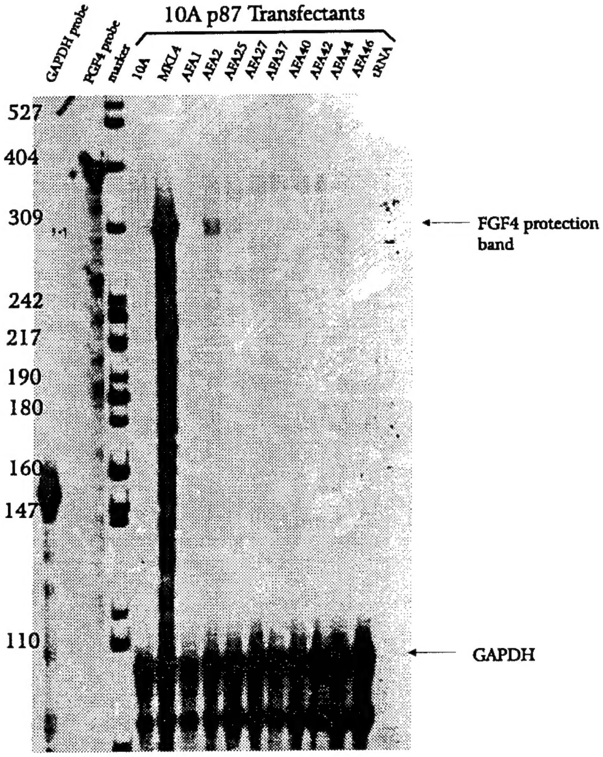


Figure 5. RNAse protection assay shows that transfected MCF-10A clones express Hyg R mRNA. β 20 are MCF-7 cells transfected with LacZ and FGF-1 β.

3. Transfection and isolation of cell lines overexpressing biologically active FGF. MCF10A cells were obtained from the American Type Tissue Collection and cultured as described in (4). We are using the modification of the calcium phosphate technique described by Chen and Okayama for these studies (5). We cotransfected the pCHCßgal *lacZ* vector that also confers hygromycin resistance (6) with one of four other vectors that all confer resistance to G418. We used 1) the pCNCEBKS1 FGF-4 vector used with MCF-7 breast cancer cells and SW13 adrenalcortical carcinoma cells (7), 2) the pCHChstFGF1ß vector that directs the expression of amino acids 1-154 of FGF-1 fused at the amino terminus to the secretory signal sequence of FGF-4, 3) the pCNCFGF1α plasmid directing the expression of amino acids 21-154 of FGF-1 or 4) the pCNCEB8 expression vector that does not contain any cDNA insert. In these vectors, expression of the respective cDNA as well as the transcription unit conferring drug resistance are both driven by a cytomegalovirus (CMV) immediate early gene promoter.

After transfection, G418 and hygromycin-resistant colonies were expanded and individual clones and polyclonal pools of clones were examined by RNAse protection assay for expression of the appropriate mRNA. We also used Southern blotting assay to examine whether the transfected genes are integrated into the MCF-10A genomic DNA. With the RNAse protection assay; we found that after transfection of MCF-10A cells with the pCHC β gal plasmid, the expression of the Hyg ^{R} gene



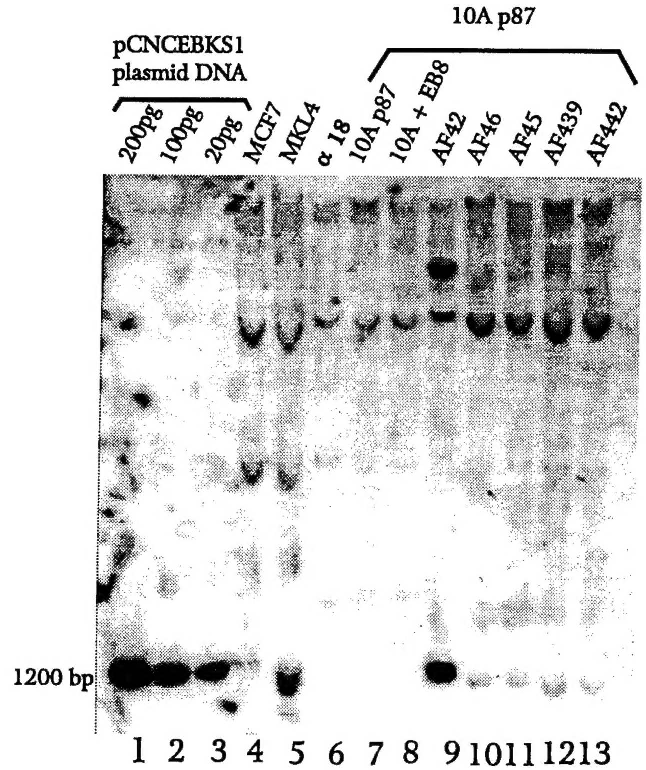


Figure 6. RNAse protection assay shows that transfected MCF-10A clones express FGF-4 mRNA. MKL-4 are MCF-7 cells transfected with LacZ and FGF-4.

Figure 7. Southern blot assay shows that FGF-4 gene is integrated into MCF-10A genomic DNA. Lane 1-3: standard amounts of EcoR I digested DNA use to show migration of the digested FGF4. Lane 4-6: MCF-7 cells alone or transfected with FGF-4 or FGF-1. Lane 6-13: MCF-10A cells alone or transfected with vector control or FGF-4 gene.

is detectable (six clones examined, all positive), but comparing to the MCF7 transfected cells, the expression level is much less. With pCNCEBKS1 (FGF4) transfectants using a FGF-4 riboprobe, we obtained three positive signals from nine clones with varying FGF-4 mRNA expression levels. However, the highest mRNA expression level of FGF-4 in the MCF-10A transfected clones is much lower than the mRNA expression level of FGF-4 in MCF-7 transfected cells. This is despite the fact that a Southern blotting assay indicates that this clone has more copies of transfected FGF-4 DNA integrated than the MCF-7 transfected cells. Therefore, the mRNA expression level of these transfected genes in MCF-10A cells is relatively low when expressed off a CMV promoter.

In an attempt to increase the expression level of these transfected genes in MCF-10A cells by finding a better promoter than CMV, we checked the transfection efficiency of MCF-10A cells with six different promoters driving a gene for G418 resistance. They are the polyoma early promoter (Py), the RSV LTR promoter (RSV), the CMV IE promoter (CMV), the adenovirus major late

promoter (Ad2) and the human metallothionine IIA promoter (hMT). The data show that the transfection efficiency of MCF-10A cells with either CMV or hMT is high. However, hMT is slightly higher and the colony size was typically larger. Similar results were obtained when MCF10A cells were transiently transfected with similar constructs where a chloramphenicol acetyl transferase gene was linked to either of these two promoters. We subcloned FGF-1 α, FGF-1 β and FGF-4 into the 3HPL vector (containing hMT promoter). We have obtained the correponding phMTFGF-1 α, phMTFGF-1 β and phMT FGF-4 plasmid constructs. We also cotransfected phMTFGF-1 α, phMTFGF-1 β with pCHC β gal *lacZ* vector with MCF-10A cells and selected for hygromycin resistant colony formation. . With pCHC β gal + phMTFGF-1 α and phMTFGF-1 β transfectants, using Hyg ^{R} riboprobe in an RNase protection assay, we obtained four positive clones which also had varying Hyg ^{R} mRNA expression level. Southern blotting assay data show that FGF-4, FGF-1 α, FGF-1 β cDNAs are integrated into the MCF-10A genomic DNA. However, when we examined the transfectants with a northern blotting assay, we were not being able to find detectable FGF-1 α or FGF-1 β expression.

Thus, we have obtained MCF-10A cells transfected with FGF cDNAs. However, the gene expression level is not satisfactory. In an attempt to solve this problem, we will use different expression systems to increase the gene expression level in MCF-10A cells. We already obtained three expression vectors (a) pHBAPrlneo (human beta actin promoter) and (b) pCI-neo (CMV promoter) that have already been used by another group with MCF-10A cells, and (c) pSG-5 (SV40 promoter). The rationale for using these two vectors is that in these vectors a chimeric intron is situated downstream of the human beta actin or CMV promoter region. The intron is located 5' to the cDNA insert in order to prevent utilization of possible cryptic 5'-donor splice sites within the cDNA sequence. Transfection studies have demonstrated that the presence of an intron flanking the cDNA insert can increase the level of gene expression in mammalian cells. Another vector pMEXneo (moloney sarcoma virus-LTR promoter) will also be tested.

CONCLUSION

1. The background screening of MCF-10A cells indicates that this cell line expresses high level of FGF2 (+++), FGFR1 (+), barely detectable FGFR2 (+/-), and does not express FGF1, FGF4 and FGFR3. At the protein level, the data indicates that a high amount of FGF2 protein exists in MCF-10A cell lysate, but barely detectable levels are present in the conditioned media.

2. The growth curve assay indicates that MCF-10A cells depend upon the presence of EGF for *in vitro* growth. In the absence of EGF, the addition of FGF1, FGF2 and FGF4 can increase cell growth but not to the levels seen with EGF addition.

3. With MCF-10A cells, we can obtain expression of the gene of interest, such as FGF4. However it has yet to be determined if this level of expression is sufficient for transformation and transfection with other expression vectors may be required to obtain such a level.

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